CHEMICAL CHANGES IN THE ADDUCTOR MUSCLE OF THE CHELIPED OF THE CRAYFISH IN RELATION TO THE DOUBLE MOTOR INNERVATION

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Work from this laboratory has shown that a number of the striated muscles of the legs of the crayfish show a typical motor innervation (1). Such muscles are innervated by two motor fibers only, each causing a different type of contraction. One of these, resulting from stimulation of the thicker fiber, is termed the "fast" contraction, while the other is called the "slow." Both anatomical evidence and physiological experiments indicate that each muscle fiber is innervated by these two axons and that both contractions occur in the same muscle fibers (2, 3).

The most striking difference between the two contractions is found in the adductor muscle of the cheliped of the crayfish. In this case, a single impulse in the thicker motor fiber causes a twitch-like contraction, and the system behaves in almost all respects as does a single motor unit of a vertebrate muscle: single shocks of different strengths give twitches of a considerable strength and a constant height, and during tetanic contraction the action currents are all of the same magnitude. In contrast to this, single shocks given to the thinner fiber produce no visible response in the muscle, but faradic stimulation causes action currents which grow in height and a contraction with a long latent period. The action currents in this case are always much smaller than those of the fast contraction.

The chemical changes occurring in the adductor muscle of the cheliped of the crayfish *Cambarus clarkii* during these two types of contraction were investigated in order to obtain evidence on two possible mechanisms by which the two contractions might occur in the same muscle fiber. In the first place, two contractile substances

might be present, a "phasic" and a "tonic" one, analogous to those supposed by Botazzi (4) in the vertebrate striated muscle, or the same substance might contract in both cases, the difference between the contractions being due solely to differences in the transmission mechanism between the nerve impulse and the contractile substance.

Methods were devised for the rapid removal of the stimulated cheliped to liquid air and for the analysis of the frozen muscle tissue. The changes in phosphate and in lactic acid content were used as indices of the chemical changes occurring.

Methods

Animals with paired normal chelipeds were used in this work. The chelipeds were cut from the animal at the ischiopodite and kept in the physiological solution described by van Harreveld (5). One cheliped served as a control for the experimental treatment of the other.

The motor nerve fiber giving the slow contraction, or that giving rise to the fast, was prepared in the meropodite in the manner described by van Harreveld and Wiersma (1). All of the motor and inhibitor fibers going to the other muscles in the cheliped were cut, as well as the unwanted motor fiber to the adductor muscle. Usually a small sensory bundle was kept intact to strengthen the bridge between the two thicker parts of the nerve bundle. The stimulating electrodes were always placed on the thicker proximal part of the nerve bundle in the meropodite.

Since it is impossible in preparing the fiber to avoid stimulation altogether, a rest period of between 45 and 120 minutes was allowed between the conclusion of preparation and the beginning of an experiment. The prepared cheliped was then fastened upon a wooden block fitted with flexible silver wire stimulating electrodes and with a rod for mounting upon a heavy support stand. The dactylopodite was tied to an isometric lever attached to the same stand. The electrodes were connected by flexible leads to a thyratron stimulator giving rectangular shocks of 0.5σ duration at various determined frequencies. The mechanical effect of the contraction resulting from the stimulus was recorded, and then the cheliped, still mounted on the block, was plunged into liquid air. Stimulation was continued during the period of removal to liquid air, this time (1–3 seconds) being included in the total time of stimulation. The control cheliped was also frozen at about the same time.

The frozen cheliped was opened, and the tissue of the adductor muscle was separated out (free of chitin and tendon) and dropped into liquid air. The tissue

fragments, together with a small amount of liquid air, were placed into the barrel of a small crusher, a greatly simplified form of that described by Graeser, Ginsberg, and Friedemann (6). It is essential to keep the tissue fragments and the pellets obtained by crushing constantly cooled with liquid air, since it was found that any thawing produces irregularities in the resting values between the muscles of paired, unstimulated chelipeds.

Phosphate Determination

A method for the removal of proteins with 25 per cent trichloracetic acid was developed, and the molybdate-stannous chloride method of Kuttner and Cohen (7) was used for the determination of phosphate in the deproteinized solution. The values are reported as milligrams per cent of phosphorus.

The frozen muscle pellet (300–500 mg.) is rapidly weighed on a torsion balance and dropped into 5.0 ml. of ice cold 25 per cent trichloracetic acid. The flask is stoppered and vigorously shaken for 15 seconds, and then ice cold water is added to make a total dilution to 25.0 ml., allowance being made for 80 per cent water content of the tissue. The diluted mixture is well shaken and set aside in an ice bath. Within 6 to 10 minutes aliquots are taken for colorimetric analysis. A red filter (Eastman Wratten filter 25A) is used in the colorimeter to avoid errors arising from the slight difference in color between standard and unknown.

It was found that, due to some property of the muscle proteins, the ordinary concentrations of 5 per cent or 10 per cent trichloracetic acid failed to give complete precipitation, a flocculent precipitate appearing in the final color mixtures. When the pellets were shaken with 25 per cent trichloracetic acid, however, the final colored solutions were clear. Before the colorimetric determination, the mixture had to be diluted to only 5 per cent trichloracetic acid in order to avoid interference in the color development. The use of ice cold water was found to minimize the effect of hydrolysis of phosphagen and so to eliminate the necessity of an extrapolation to a zero time value. No catalysis of the hydrolysis of the phosphagen by acid-molybdate was observed.

Analyses carried through the complete procedure gave an average recovery of 99 per cent of added phosphate. The method of protein precipitation described has also been found suitable for dealing with *Astacus trowbridgii* and with *Pagurus ochotensis*.

Lactic Acid Determination

The protein precipitation procedure used was the modification of the zinc hydroxide method of Somogyi described by Graeser, Ginsberg, and Friedemann (6), with the exception that, since no sugar determinations were made, glucose was not added to the zinc sulfate-sulfuric acid solution. For the removal of bisulfite binding substances the copper-lime precipitation was used.

For the determination of lactic acid in the copper-lime filtrate a modification of the direct distillation procedure of Friedemann and Graeser (8) was made. Following the work of Avery, Kerr, and Ghantus (9), reduced volumes of sample and of reagents were used. The distillation apparatus was modelled after the micro-distillation apparatus of Lehnartz (10). The manganous sulfate-sulfuric acid reagent of Friedemann and Graeser was found to be as effective for the conditions adopted as the stronger reagent of Avery, Kerr, and Ghantus, and potassium permanganate was found satisfactory as the oxidizing agent.

An average recovery of 96 per cent was obtained with known lactic acid solutions carried through the entire procedure.

RESULTS

Examination of Possible Interfering Factors

The suitability of the use of one cheliped to obtain the resting value for the other was investigated. In Table I are summarized the results of a number of experiments made to compare the resting value levels of phosphate and of lactic acid in the adductor muscles of paired unstimulated chelipeds. The difference between the chelipeds of one animal is small, so it is clear that one cheliped may be used as the control for the other.

There are several possibilities of production of phosphate and of lactic acid from stimuli other than those controlled in an experiment. The factors considered have been preparation of the nerve, rest in physiological solution, and the freezing of the cheliped in liquid air. The most important of these, in view of the unsymmetrical stimulation involved, is the preparation of the nerve bundle.

A series of experiments were performed in which the nerve bundle of one of a pair of chelipeds was given the maximum stimulation encountered in preparation. Both were then allowed to rest for the usual time, frozen, and analyzed. The same differences were found

TABLE I

Difference in Phosphorus and Lactic Acid Values between the Adductor Muscles of Paired, Unstimulated Chelipeds

Analysis for.....	Phosphorus			Lactic acid		
Milligrams per cent found in.....	Left muscle	Right muscle	Difference	Left muscle	Right muscle	Difference
Values found	51	57	±6	19	26	±7
	49	54	5	26	18	8
	40	39	1	34	29	5
	53	52	1	17	14	3
	55	53	2	19	20	1
	47	45	2	28	30	2
	51	49	2	25	30	5
	55	49	6	28	25	3
	51	48	3	14	15	1
	44	46	2	14	20	6
	47	44	3	17	22	5
	60	56	4	20	22	2
	58	60	2			
	51	51	0			
	60	58	2			
	59	59	0			
	44	48	4			
	51	53	2			
	48	53	5			
Mean value.....	51.1 ± 1.2	51.2 ± 1.2		21.8 ± 1.9	22.2 ± 1.5	
No. of values.....	19			12		

as are recorded in Table I for paired unstimulated chelipeds; therefore, the phosphate or lactic acid formed has disappeared on resting.

In a series of experiments in which one cheliped was dropped into liquid air immediately upon cutting from the animal, with the other being allowed to stand in physiological solution for periods up to 2 hours, there was found only a small

increase in phosphate and lactic acid in the latter. This amount is within the limits of variation noted for the resting values between paired unstimulated chelipeds.

It was observed that an unstimulated cheliped would sometimes have the dactylopodite in the closed position after liquid air treatment and sometimes not (the stimulated chelipeds were always closed). For many of the experiments on control values (Table I), a record was kept of this position of the dactylopodite. No correlation with the analytical results was found; and it was concluded that if the closing of an unstimulated cheliped is indicative of a real contraction, this contraction adds no measurable amount to the production of phosphate or of lactic acid. It is more likely that the closed position is indicative of some mechanical effect of freezing. Although Meyerhof and Lohmann (11) are of the opinion that freezing of the tissue causes a large breakdown of the phosphagen, the present work shows either that such an effect does not enter or that it is small and so uniform as not to enter into the present considerations. It may be that some thawing occurred in the work of Meyerhof and Lohmann, a condition conducive to high results.

Stimulation of the Isolated Motor Axons at Determined Frequencies

It is of special importance to note that at stimulation frequencies of 50 shocks per second for the fast contraction and at 200 for the slow, the resulting contractions are very much alike. The maximum tensions developed in different preparations are in the main very similar and are reached within 2 seconds, and both kinds of contractions have a similar fatigue. This is in accord with the findings of Wiersma and van Harreveld (3), who obtained evidence that in one cheliped these two contractions are almost identical in strength and in speed. In contrast to this, the slow contraction at 50 shocks per second shows a much more gradual rise in tension before the maximum is reached (about 15 seconds); and the maximum tension, though of the same order, is on the whole less. A few attempts were made to investigate the fast contractions arising at a stimulation frequency of 200 per second, but the very quick drop in tension after the maximum had been reached made impracticable their use for this investigation.

In Table II are summarized the results of the stimulation experiments. The string to the lever was cut as soon as the contraction began to drop from the maximum. In the case of the slow contractions obtained by stimulation at a frequency of 50 shocks per second,

fatigue is very much postponed, and it was possible to continue these for much longer periods than the fast at 50 or the slow at 200 shocks per second. It will be seen from the table that although the slow contraction at 50 shocks per second was of much longer duration, no significant increase in the amount of phosphate, and only a small increase of the lactic acid, was found. The increases in the amount of phosphate during contractions arising from stimulation of the fast system at 50 per second and of the slow at 200 for the same period were closely alike, both showing the same very definite rise above the base level. The lactic acid formation was also definitely larger than in the slow at 50 per second, but there was a difference which may be significant in that the slow at 200 seems to form less than the fast at 50 per second.

It is to be seen that practically all of the contractions of the fast at 50 and of the slow at 200 per second done for the lactic acid measurements are of a duration of less than 15 seconds. These experiments were performed at another time of the year than those on phosphate, and all preparations showed at this time a quicker fatigue. A similar difference is present in the slow contractions at 50 per second: in the lactic acid determinations, the average duration of contraction is 30 seconds, whereas the mean value for the phosphate determinations is 80 seconds.

No way could be found of correlating the tensions produced in the contractions of the muscles of the different animals with the chemical changes found. The nature of the attachment of the adductor muscle to the chitin and to the tendon makes extremely difficult a complete isolation of the liquid air frozen muscle. Thus the estimation of any value for the size of the muscle, a factor of great importance with regard to the total tension developed, was not possible.

A factor which is not clear from the tables presented is the relation between phosphate and lactic acid and the stimulation time. In order to show that such a relation is indeed present under our experimental conditions, the chemical changes after a determined number of twitches were measured. No preparation of the nerve fibers was necessary in these experiments (although carried out in a number of cases), since stimulation of the slow fiber at these frequencies has hardly any effect mechanically and (as is evident from the determina-

TABLE II

Formation of Phosphate and of Lactic Acid during Slow and Fast Contractions

Preparation		Analysis for							
		Phosphate (as phosphorus)				Lactic acid			
Fiber	Frequency of stimulation (shocks per second)	Stimulation time	Milligrams per cent found in			Stimulation time	Milligrams per cent found in		
			Control muscle	Stim. muscle	Difference		Control muscle	Stim. muscle	Difference
		sec.				*sec.*			
Slow	50	51	49	43	−6	32	28	24	−4
		62	58	55	−3	17	23	21	−2
		122	59	57	−2	22	17	19	+2
		32	58	61	+3	15	15	18	+3
		92	49	53	+4	27	23	26	3
		62	51	56	5	31	24	28	4
		47	48	53	5	17	28	33	5
		92	61	67	6	62	12	22	10
		122	38	45	7	38	13	26	13
		122	45	52	7	62	26	41	15
						16	18	34	16
						21	24	42	18
Mean value.....			51.5±2.1	53.9±2.1			21.0±1.6	28.0±2.2	
Difference in mean values.....			2.4±3.0				7.0±2.7		
Fast	50	12	55	59	+4	8	17	24	+7
		11	57	65	8	11	13	20	7
		9	41	51	10	10	21	28	7
		12	56	67	11	4	30	43	13
		17	56	68	12	7	21	36	15
		18	61	74	13	12	23	42	19
		17	57	71	14	8	21	41	20
		17	59	74	15	8	19	40	21
		18	44	63	19	9	16	39	23
		15	40	62	22	8	27	53	26
		10	55	78	23				
		32	51	75	24				
		17	56	85	29				
Mean value.....			52.8±2.1	68.5±2.4			20.5±1.5	36.7±2.9	
Difference in mean values.....			14.7±3.0				16.2±3.3		

TABLE II—*Concluded*

Preparation		Analysis for							
		Phosphate (as phosphorus)				Lactic acid			
Fiber	Frequency of stimulation (shocks per second)	Stimulation time	Milligrams per cent found in			Stimulation time	Milligrams per cent found in		
			Control muscle	Stim. muscle	Difference		Control muscle	Stim. muscle	Difference
		sec.				*sec.*			
Slow	200	17	54	64	10	7	17	18	1
		17	58	70	12	8	12	16	4
		17	56	69	13	10	16	21	5
		17	41	60	19	18	17	24	7
		16	60	82	22	8	18	30	12
						11	18	31	13
						11	26	41	15
						9	22	39	17
						7	31	49	18
						6	21	47	26
Mean value.....			54.1±3.3	69.7±3.4			19.9±1.6	31.7±3.3	
Difference in mean values.....			15.6±4.8				11.8±3.7		

TABLE III

Phosphate and Lactic Acid Formation on Single Shock Stimulation

(Frequency = 1, 3, 6, or 12½ shocks per second)

Total number of twitches	Milligrams per cent increase in			
	Phosphorus		Lactic acid	
	No. of values	Average value	No. of values	Average value
15– 50	3	7	9	7
50–100	8	11	7	8
100–150	2	13	4	12
150–200	3	23	2	20
200–400	3	23	—	—

tions upon slow preparations at 50 per second) certainly causes no appreciable chemical changes. The results are given in Table III. It will be seen that the production of phosphate gradually rises with

an increasing number of twitches and that the lactic acid formation is somewhat similar, although not so clearly demonstrated as that of the phosphate.

Phosphagen Phosphate

Since the amount of phosphate found in the stimulation experiments was, on the average, no more than 16 mg. per cent phosphorus (highest value, 29 mg. per cent) above the resting level, it was of interest to

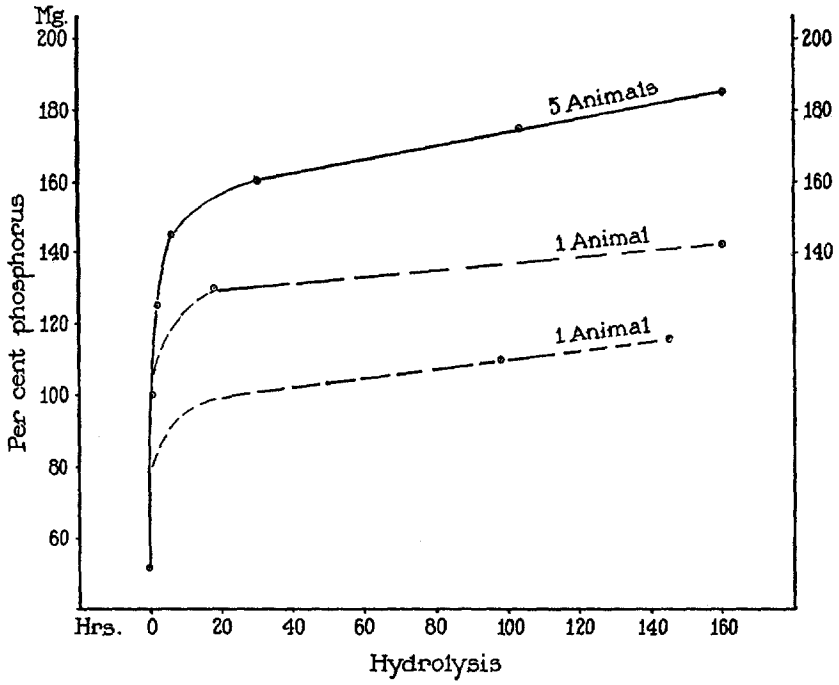

FIG. 1. Hydrolysis of phosphate compounds in the adductor muscle.

determine the fraction of the total available phosphagen represented by this amount. A number of experiments were made in which pairs of chelipeds were frozen in liquid air, with or without stimulation of one of the pair. The initial phosphate was determined, and the mixture of tissue and 5 per cent trichloracetic acid was left standing at 35°C., determinations being made at intervals.

The results are plotted in Fig. 1, the initial portion of the curve for one experiment being shown in detail in Fig. 2.

In the group of five animals giving closely concordant data, it will be seen that during the first 2 hours the hydrolysis proceeds rapidly, a level of 130–135 mg. per cent phosphorus being reached. The rate then decreases, and after about 30 hours (level of 160 mg. per cent) a new, much slower rate is followed. At the end of 160 hours the final levels for the five pairs of chelipeds are 198,197; 181,176; 189,185; 176,174; 184,184: the average is 185 mg. per cent.

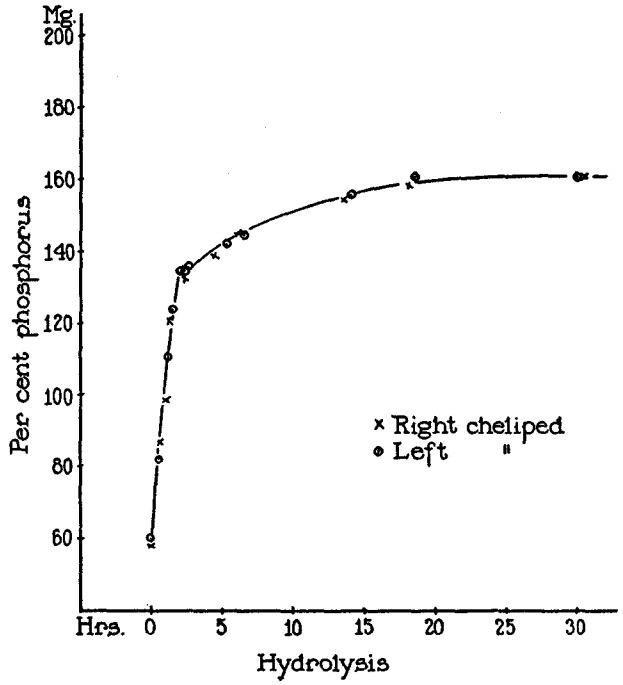

FIG. 2. Production of phosphate on hydrolysis of the tissue from the paired claws of one animal.

If the hydrolysis during the first 2 hours is considered as being that of the phosphagen and the remainder is assumed to be that of other more or less readily hydrolyzable phosphate compounds, the representative phosphagen level should be about 140 mg. per cent. With a resting level of phosphate at 50 mg. per cent phosphorus, the amount of available phosphagen is in the order of 90 mg. per cent phosphorus.

Thus, on the average, less than one-fifth of the available phosphagen was found hydrolyzed on contraction, at the most one-third.

Experiments in which one of the pair of chelipeds was stimulated showed at the beginning of the hydrolysis the accustomed difference in the phosphorus level, but this difference had disappeared at the end of the first 2 hour period.

Of the two deviating animals, one had a phosphagen level at about 110 mg. per cent and a final level at 142 mg. per cent phosphorus; the other, only 90 and 116 mg. per cent respectively. Also in these cases, the paired chelipeds gave closely similar figures.

DISCUSSION

The present investigation was undertaken in order to compare the chemical changes in the fast and the slow contractions and to correlate this information, if possible, with existing physiological data. The results obtained clearly show that the chemical changes involved in the two types of contraction are of the same order if the mechanical effects are comparable, and that they are distinctly different if the stimulation is such as to produce quite different mechanical effects. Since equivalent changes accompany equivalent action, it is necessary to assume that the two types of contraction do not differ in the mechanism of the chemical changes involved, which is strongly indicative of the presence of only one contractile substance.

The evidence obtained thus strengthens the conclusions arrived at by anatomical and physiological experiments (2, 3); namely, that each muscle fiber is innervated by both the fast and the slow axon and that both contractions occur in the same muscle fiber. The difference between the two contractions then lies in the modes of transmission at the neuromuscular junction.

For the present considerations more emphasis has been placed upon the phosphate values, for it is generally considered that the phosphate stands in a more directly integral position in the cycle of chemical changes in muscle than does the lactic acid. That only a fraction of the available phosphagen is found broken down to phosphate after contraction is of significance towards understanding the individual variations observed between animals. This fraction is probably more

apparent than real, its exact magnitude depending upon the balance between breakdown and resynthesis. Also, the supply of total phosphate and of available phosphagen is variable between animals.

The fact that no catalysis of the hydrolysis of the phosphagen in acid-molybdate solution was observed in the course of the phosphate determinations indicates that the phosphagen of *Cambarus clarkii* is arginine phosphoric acid rather than the creatine compound. This conclusion is substantiated by the results of previous investigators (Kutscher (12), Eggleton and Eggleton (13), Meyerhof and Lohmann (14), Schutze (15)) on the isolation of the phosphagen of crustaceans.

The resting value for phosphate of Schutze on *Astacus fluviatilis* (about 90 mg. per cent phosphorus) is higher than that found in the present work on *Cambarus clarkii*, as are his maximal values (240 mg. per cent). The high resting value probably is due to injury of the muscle tissue during preparation for analysis. Meyerhof and Lohmann (11) give a much lower resting value (about 35 mg. per cent) for *Astacus*, but their maximal values are only about 100 mg. per cent. The discrepancy in the breakdown phosphate values no doubt lies in the conditions of the hydrolysis, and that the values of Meyerhof and Lohmann are lower than those of the present work may be due to a difference between *Astacus* and *Cambarus*. In this connection it is of interest that the available phosphagen for the latter amounts to 65 per cent of the sum of resting phosphate plus that from phosphagen, a figure in agreement with the 60–75 per cent noted by Meyerhof and Lohmann for *Astacus*.

The very high resting lactic acid values of Schutze (average 322 mg. per cent for 9 values) are not in accord with the present data. However, the few values given by Meyerhof and Lohmann are of the same order as the present ones, and it may be that the analytical method used by Schutze gave apparent high results.

SUMMARY

An investigation has been made of the phosphate and lactic acid changes in the adductor muscle of the cheliped of the crayfish *Cambarus clarkii* upon stimulation of the isolated axons for the fast and slow contractions at determined frequencies. The data obtained point to the following conclusions:

1. When the mechanical effects of the two types of contraction are the same, the chemical changes are of the same order. If the mechanical effects are different, the chemical changes likewise are not equivalent. This is especially to be seen in the case of stimulation at 50 shocks per second: a slowly rising, long continued, strong slow contrac-

tion takes place with no apparent change in the phosphate content; a quickly rising fast contraction occurs with a large increase in the phosphate.

2. Since equivalent chemical changes accompany equivalent mechanical action, the two types of contraction do not differ in the essential mechanism of the chemical changes involved, and only one type of contractile substance is present.

3. Even when a contraction has taken place to the maximum extent obtainable, only enough phosphate is found to correspond to one-fifth to one-third of the available phosphagen.

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